

"METHODS FOR PLANT TRANSFORMATION AND REGENERATION"

TECHNICAL FIELD

The invention relates to methods for the genetic transformation of plants and methods for regeneration of transgenic plants. In particular the invention relates to methods of transformation and/or regeneration of transgenic poppy plants.

BACKGROUND ART

The importance of the plants of the poppy family, for example *Papaver* and *Eschscholtzia* species, as a commercial source of medicinal opiates and related compounds is well known, and requires little introduction. The demand for these plant products is high.

Suitable agricultural land for commercial poppy growing is limited. Poppies need fertile, free draining soil which is not overly acidic. To reduce the build up of disease in commercially grown poppies, crops must be grown with at least a three year rotation (e.g. at least two to three different crops should be grown in the soil before poppies are grown again). There are other limitations such as topography and availability of water for irrigation. Presently in some areas, the crop area is probably close to the sustainable level - if higher yield of poppy products are desired then it will be necessary to either shorten the rotation or expand the area under cultivation to include marginal soil types. It is expected that employing these less than desirable practices will impact on factors such as yield and quality and produce undesirable related outcomes such as soil erosion and so on.

The alkaloid content in harvested poppy straw in Tasmania, for example, is generally in the range of 1.2% to 2.7% on a dry weight basis. The financial return to the growers is calculated on the basis of the alkaloid content. Thus, high alkaloid content plants mean that the poppy industry can compete with alternative crops which might potentially be grown in the same soils. High alkaloid content in the poppy crops makes the whole industry more competitive. Fewer hectares of crops would need to be grown to produce the same amount of alkaloid, and costs associated with harvesting, transport, storage, extraction and waste disposal would be reduced. Thus, high alkaloid producing

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poppies are highly desirable to growers, pharmaceutical companies and consumers of refined poppy products.

Conventional plant breeding has produced significant advances in poppy alkaloid contents over the last two decades. However, it appears that the amount of additional improvement possible through conventional breeding is limited.

Genetic transformation of poppies offers the opportunity to improve the alkaloid content of poppy crops and poppy straw. This could occur through a number of ways, including:

- enhancement of activity of enzymes at "bottlenecks" in the alkaloid synthetic pathway;
- blockage of undesirable "side reactions"; and
- blockage of the synthetic pathway so that certain desirable alkaloids accumulate (e.g. thebaine, codeine, oripavine etc.).

These types of improvement would thus allow the industry to continue to expand without increasing the area of crops grown. They would also introduce efficiencies throughout the production process.

As well as increasing the yield of desirable plant products, it is desirable to use related biotechnological procedures to introduce herbicide resistance into poppies. At present herbicide control of weeds in poppy crops is difficult and costly. Herbicides are not developed specifically for poppies and the spectrum of weed control of any one herbicide is not very wide. Thus programs of herbicides are applied involving a number of different products tank-mixed and applied in sequence. Development of a herbicide resistant poppy will enable the use of a herbicide with a wider margin of crop safety, and a wider weed spectrum than currently available. The cost of such weed control is expected to be significantly less than presently involved.

Genetic transformation may also be used to introduce other genes into poppies to impart commercially desirable properties, for example, resistance to disease, resistance to acid soil and resistance to insects and other pests.

Despite the desirability of such transformations, it has so far proved difficult to produce viable transgenic poppies. Attempts using conventional methods to introduce

specific gene sequences encoding for certain properties and subsequent regeneration of transgenic poppies with predictable properties have thus far been mostly unsuccessful.

Thus there exists a need to develop methods for stably introducing genetic material into a plant which results in a plant which is viable and which possesses the  
5 desired traits.

It is an object of the present invention to overcome or ameliorate at least one or more of the abovementioned deficiencies in the prior art, or provide a useful alternative.

### SUMMARY OF THE INVENTION

The present applicant has found that there is an unexpected and  
10 rapid rise in the pH of the culture medium when plant cells, such as poppy cells, are cultured. This rise in pH is observed around poppy cultures including initial explants (eg. seedling hypocotyls), undifferentiated callus, and callus regenerating via somatic embryos or shoots. This feature of plant cultures, such as poppy cultures, is at least in part responsible for lack of success in regenerating poppy plants and  
15 recovering transgenic poppy plants.

Throughout this specification, "type I callus" is translucent callus which can be colourless or brown, and is composed of large vacuolate cells. "Type II callus" is white to brownish opaque callus, composed of small cytoplasmic cells and having the capacity to form somatic embryos and meristemoids.

20 The term "pH measurements" in the context of the present specification refers to pH measurements taken using a strip of agar from the medium in which the cultured tissue is growing, homogenising in water and reading the pH by conventional means. It will be clear to those skilled in the art that changes of pH in the immediate microenvironment of the plant tissue or cells may be more extreme than those measured  
25 at some distance (usually 1 cm) from the cells.

According to a first aspect, the invention provides a method of producing a transgenic plant comprising the steps of:

1) introducing exogenous genetic material into plant material in the presence of a buffering agent which prevents, reduces the rate of or delays the rise in pH of culture  
30 medium or plant material.

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2) culturing said plant material in the presence of a buffering agent which prevents, reduces or delays the rate of rise in pH of the culture medium or plant material; and

3) regenerating a transgenic plant from said plant material.

5 According to a second aspect, the invention provides a method of transforming a plant comprising the step of introducing exogenous genetic material into plant material in the presence of a buffering agent which prevents, reduces the rate of or delays the rise in pH of culture medium or plant material.

According to a third aspect, the invention provides a method of producing a  
10 transgenic plant from plant material harbouring exogenous genetic material comprising the steps of:

1) culturing said plant material in the presence of a buffering agent which prevents, reduces the rate of or delays the rise in pH of the culture medium or plant material; and

15 2) regenerating a transgenic plant.

Preferably the plant is an alkaloid producing poppy plant and even more preferably the plant is selected from the *Papaver* species or *Eschscholtzia* species. The most preferred species is *Papaver somniferum*.

Preferably the plant material is derived from seeds, imbibed seeds or seedling  
20 parts of the plant. Preferably the plant material is selected from the group comprising seed explant, seedling explant, type I callus, type II callus, somatic embryogenic callus and any culture which gives rise to somatic embryos or shoots. Alternatively the plant material could be derived from plant tissues such as leaves, stems, roots or flowers.

Preferably, the buffering agent used prevents or delays the rapid rise in pH of the  
25 culture medium in which the plant material is transformed or in which the transformed plant materials is being regenerated into a transgenic plant. More preferably the pH is maintained between pH 5.5 - 6.5.

Preferably the buffering agent is selected from the group consisting of 2-[N-morpholino]ethane sulfonic acid buffer (MES), N-[2-acetamido]-2-iminodiacetic acid  
30 buffer (ADA) and bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane buffer (BIS-TRIS) or a solution containing ammonium and nitrate ions in a predetermined ratio. In

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the light of the teaching of this disclosure those skilled in the art will be able to identify other buffers suitable for use in the method.

Preferably the exogenous genetic material is introduced into plant cells by a plant transformation agent, most preferably *Agrobacterium tumefaciens*. In another preferred embodiment the exogenous genetic material may be introduced using a mechanical method such as microparticle bombardment. Other methods of introducing exogenous genetic material into plant material would be clear to those skilled in the art.

The genetic material may be DNA or RNA and may encode a gene or a fragment of a gene, or it may represent antisense nucleotide sequences of endogenous genes.

10 Preferably the exogenous genetic material encodes a mRNA or protein that confers on the transgenic plant a property selected from the group comprising: increased alkaloid yield relative to the native alkaloid producing plant, increased herbicide resistance relative to the native alkaloid producing plant, increased soil acidity tolerance relative to the native alkaloid producing plant, increased disease resistance relative to the native  
15 alkaloid producing plant, increased insect resistance relative to the native alkaloid producing plant, increased growth rate relative to the native alkaloid producing plant, improved flowering properties relative to the native alkaloid producing plant, increased frost tolerance relative to the native alkaloid producing plant and altered alkaloid proportions relative to the native alkaloid producing plant. Most preferably the  
20 exogenous genetic material encodes a mRNA or protein that confers on the transgenic poppy the property of altered alkaloid proportions relative to the native alkaloid producing plant. When the exogenous genetic material encodes a mRNA or protein that confers on the transgenic poppy the property of herbicide resistance, preferably the herbicide resistance is Basta herbicide resistance, glyphosate resistance, bromoxynil  
25 resistance or sulfonylurea resistance.

Preferably the exogenous genetic material is comprised in a DNA construct based on the binary vector pPZP, most preferably pTAB101 with 35S 5':*pat*:35S 3'. In another preferred embodiment the binary vector is pBSF16. In a particularly preferred embodiment, the binary vector is pPOP5.

30 According to a fourth aspect the invention consists in a transgenic plant prepared by the method of any one of the preceding aspects.

Preferably the plant is an alkaloid producing poppy plant and even more preferably the plant is selected from the *Papaver* species or *Eschscholtzia* species. The most preferred species is *Papaver somniferum*.

Unless the context clearly requires otherwise, throughout the description and the  
5 claims, the words 'comprise', 'comprising', and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 Shows the plasmid pTAB101, which is a preferred vector for  
10 introducing exogenous genetic material in accordance with the present invention.

Figure 2 Shows pBSF16, which is an alternative binary vector for introducing exogenous genetic material in accordance with the present invention.

Figure 3 Shows pPOP5, which is an alternative binary vector for introducing exogenous genetic material in accordance with the present invention.

15 Figure 4 Effects of various  $\text{NO}_3^-/\text{NH}_4^+$  ratios in culture medium on type I and type II callus weight.

Figure 5 Effects of various buffering agents on pH of the callus growth medium.

Figure 6 Effects of various MES buffer concentrations on pH of the callus growth medium.

20 Figure 7 PAT (phosphinothricin acetyl transferase) Assay. The arrow indicates the radioactive acetylated PPT band resulting from PAT enzyme activity. 1 and 9: A transgenic tobacco extract as a positive control. 2 and 10: A non-transgenic control-poppy. 3 - 8: Various primary transgenic poppy plant extracts, from plants transformed with the pTAB101 binary vector.

25 Figure 8 Western blot of seed from transgenic line 45-25, transformed with pBSF16. SSA standards are various amounts of sunflower seed albumin.

C. is control non-transgenic seed extract. T, is transgenic seed extract. The signals results from specific binding of an antiserum to the sunflower seed albumin protein.

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## DESCRIPTION OF THE PREFERRED EMBODIMENT

The method comprises in part the use of conventional methods of plant transformation and regeneration of transgenic plants but in addition includes steps which improve the conventional methods by stabilising the pH of the medium by way of either preventing or delaying the rapid rise in pH of the culture medium or the plant material. In particularly advantageous variants of the methods of the present invention the pH is stabilised within the range of pH 5.5-6.5 during both transformation and regeneration of transgenic plants.

In most plant species studied, the introduction of recombinant DNA and the culture of seedling explants on standard tissue culture media is not accompanied by a large rise in pH, however, this hitherto unexpected phenomena has been identified in poppy cultures. It has been observed by the applicants that there is an unexpected and rapid rise in the pH of tissue culture media used to support the growth of *Papaver somniferum* tissues or cells. This phenomenon may also apply to other plants and this can be simply ascertained by those skilled in the art. This very rapid and substantial rise, for example, from pH 5.6 to pH>6.4 in the immediate area around a Type II callus in B50 medium within 30 minutes, rising ultimately to pH 8.7, has been identified as a major cause of poor growth and the difficulty in producing transgenic poppies.

The preferred medium for transformation and culturing of transgenic plants is 19D (also referred to as the Callusing Medium) buffered with MES. However, any method or medium modification which for preference results in the medium pH remaining within the range of pH 5.5-6.5 is suitable for the method described herein, including, addition of MES buffer (eg 10 mM), addition of BIS-TRIS buffer (eg 10 mM), addition of ADA (eg 10 mM), modifying the ammonium and nitrate ion amounts and ratio in the medium (such as for example  $\text{NO}_3^-/\text{NH}_4^+$  of 1:3, total N of 30 mM).

In addition to helping control the medium pH during culture, the buffering agents may produce direct or indirect benefits to the process such as improving *Agrobacterium*-mediated gene transfer, type II callus formation or somatic embryo formation and development. A number of authors have indicated the significance of pH on T-DNA transfer by *Agrobacterium* (Holford et al, 1992; Fenning et al 1996 a,b; Li and Komatsuda, 1995).

The preferred exogenous genetic material used in transformation is the binary vector TAB101 containing 35S 5':*pat*:35S 3'.

Another preferred exogenous genetic material is the binary vector BSF16.

A further preferred vector is pPOP5 which has two genes in the T-DNA: the *pat* gene conferring Basta (or PPT) resistance; the *Papaver somniferum* P450 reductase gene which enables the cytochrome P450 reductase enzyme to accumulate to higher levels in the transgenic tissues; this enzyme donates electrons to reconstitute the cytochrome P450 complex which can be rate limiting for a number of cytochrome P450-dependent enzymes involved in morphine biosynthesis; the transgene is therefore expected to increase the biosynthesis of morphinan alkaloids.

The *pat* gene serves two purposes, as a selectable marker *in vitro* and as the herbicide resistance gene in the transgenic plant. As a selectable gene, it enables selection of transgenic cells in the culture using Basta herbicide or the active ingredient, glufosinate ammonium or phosphinothricin (also known as PPT). Those skilled in the art will know that alternative selectable genes could be employed such as those conferring hygromycin resistance, kanamycin resistance or spectinomycin resistance.

It will also be known to those skilled in the art that it is possible to introduce exogenous genetic material coding for more than one desirable property. For instance, the pBSF16 vector has three genes in the T-DNA: the *bar* gene conferring Basta (or PPT) resistance; the sunflower albumin gene, SF8g, which enables a novel sunflower seed albumin to accumulate in the seeds of the transgenic plant; the *GUS* reporter gene, which encodes  $\beta$ -glucuronidase and enables the detection of transgenic tissues.

## EXAMPLES

### Example 1. Plant material

The genotypes of *Papaver somniferum* used were C 046-3-5, C 058, C 060, C 048-6-14-64 and D 233 (Norman) obtained from Tasmanian Alkaloids. Seeds are surface sterilised by washing for 30-60 seconds in 70% ethanol then in 1%(w/v) sodium hypochlorite solution plus 1-2 drops of autoclaved Tween 20 or Triton X for 20 minutes with agitation. Seeds are rinsed three to four times in sterile distilled water or until no smell of bleach remains and placed on 90 x 25 mm Petri dishes containing B5O medium (see below). Dishes are sealed with Micropore tape and are usually stored at 4°C for 24



to 48 hours. Seeds are germinated at 24°C in a 16 hour light-8 hour dark cycle.

Hypocotyls are excised from seedlings after 7-8 days of culture and are cut into 3-6 mm explants (usually 1-3 explants per seedling) and used in transformation experiments.

Example 2. Tissue culture media and conditions

- 5 B5O medium consists of B5 macronutrients, micronutrients, iron salts and vitamins (Gamborg et al, 1968), 20g/L sucrose using 0.8% Sigma Agar as the gelling agent. pH is adjusted with 1M KOH to pH 5.6.

Callusing Medium (also referred to as 19D) is identical to B5O except that it includes 1mg/L 2,4 dichloro phenoxy acetic acid (2,4-D).

- 10 19D may be buffered with the appropriate buffering agent selected from MES, BIS-TRIS, ADA or modified  $\text{NO}_3^-/\text{NH}_4^+$  ionic ratios.

Medium #7 is 19D but modified in  $\text{NO}_3^-/\text{NH}_4^+$  content to achieve a ratio of 1:3 with a total N of 30 mM.

Buffering agents are added prior to autoclaving.

- 15 All media is autoclaved at 121°C for 20 minutes.

Suitable antibiotics, such as timentin, are added to all media after autoclaving and cooling to 55-65°C.

Explant and type I callus cultures are grown in Petri dishes sealed with Micropore tape at 24°C. Type II callus and somatic embryos are cultured at 18-21°C.

20 Example 3. Bacterial strains and binary vectors

- The disarmed *Agrobacterium tumefaciens* strains AGLO and AGL1 (Lazo et al., 1991) are used in transformation experiments. DNA constructs are based on the binary vector pPZP201 (Hajdukiewicz et al, 1994), e.g. pTAB101 (see Fig. 1) with 35S 5':*pat*:35S 3'. *Agrobacterium* strains are maintained in glycerol at -80°C or on LB agar plates plus appropriate selection at 4°C. Fresh cultures are grown overnight at 28°C in 25 10 mL MG broth (Garfinkle and Nester, 1980) without antibiotics. This *Agrobacterium* suspension is diluted to approximately  $5 \times 10^8$  cells  $\text{mL}^{-1}$  ( $\text{OD}_{600} = 0.25$ ) for use in transformation experiments.

Example 4. Transformation and embryogenesis

Hypocotyls are excised from seedlings and immediately inoculated by immersion in liquid *Agrobacterium* culture for 10-15 minutes. Explants are then transferred directly to 19D, with or without buffering agent, or medium #7. After four to five days co-cultivation explants are washed in sterile distilled water, until the water is clear of *Agrobacterium*, blotted on sterile filter paper and transferred to 19D, with or without buffering agent, or medium #7 containing 150 mg/L Timentin plus 10 mg/L PPT (phosphinothricin, the active ingredient of Basta herbicide). Timentin is included to control *Agrobacterium* overgrowth and it will be clear to those skilled in the field that suitable alternative antibiotics or agents may also be used. Explants are transferred to fresh 19D, with or without buffering agent, or medium #7, at three weekly intervals. They initially produce friable brownish type I callus and may subsequently form small regions of very white, compact embryogenic callus (type II) by about 7-8 weeks culture.

Type II callus is transferred to B5O containing 150 mg/L Timentin plus 10 mg/L PPT and cultures are transferred to fresh medium every three weeks. Meristemoid/embryo development usually occurs after one or two periods on B5O medium and are seen from about 14-16 weeks total culture time.

Plantlet development from embryos is slow and may require a further 3 months in tissue culture before shoot and root growth is sufficient to ensure successful transplantation to soil.

Example 5. Importance of pH buffering

If the initial pH of the medium is 5.8 and buffering agent is omitted, the pH of poppy cultures rapidly rises to pH 8.0 or higher when the callus mass reaches about 1 cm diameter. Fresh agar-solidified B5-based medium adjusted to pH 5.6 rose to pH > 6.4 in the immediate area around type II callus within 30 mins. The inclusion of chlorophenol red in the medium was sometimes used to observe these localised increases in pH; the medium turns purple at pH 6.4. The whole plate was pH > 7 within 24 h. At the end of the culture period pH values were measured at 8.7. This rapid rise in pH results in very poor growth which is not compensated for by frequent changes of medium. The rapid rise was significantly delayed even by 2.5 mM MES, but 10 mM MES is preferred to adequately buffer the medium and support improved growth over the 3 week subculture

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With *Agrobacterium* co-cultivation and PPT selection, the unbuffered medium (19D) did permit unacceptable rises in pH (Fig. 5 C) and accumulation of black or brown pigments even though the amounts of tissue involved were very small. The pH was controlled by the addition of 10 mM of the buffers MES, ADA or Bis/Tris and the pigmentation was less severe. Unacceptable increases in medium pH were also controlled by a buffering strategy based on an alteration of the nitrate to ammonium ratio in medium #7 which has  $\text{NO}_3^-:\text{NH}_4^+$  of 1:3 molar. The rise in pH was most extreme when explants were under PPT selection but had not been treated with *Agrobacterium* (Fig. 5 A). After the first three weeks these cultures showed no growth as expected. Without PPT selection and without *Agrobacterium* cocultivation (Fig. 5 B) the upward pressures on pH were not evident until the third culture period when the amount of tissue had increased.

Adequate control of pH under the conditions used was achieved with 10 mM MES (Fig. 6 A, B, C). 50 mM MES in the absence of *Agrobacterium* and no PPT permitted healthy unpigmented growth. However, in the presence of *Agrobacterium* and with PPT selection, 20 and 50 mM MES were less effective gave no growth.

- 5 Pooled data over a number of experiments, using two poppy cultivars and a number of binary plasmids are shown in Table 1. These experiments are all under PPT selection.

TABLE 1 Pooled data from 8 experiments, using two poppy cultivars and a number of binary plasmids. These experiments are all under PPT selection.

Medium for initiation of culture	Medium for later stages of the transformation	Number of explants	Number of putative transgenic plants	Number of independent transgenic events
19D (no buffer)	19D (no buffer)	623	1	1
19D (no buffer)	19D + 10mM MES	300	21	1
19D + 10 mM MES	19D + 10 mM MES	968	≥60	≥14

- 10 TABLE 2 One particular experiment as an example is shown in Table 2. Measurements were made at 9 weeks from initiation. All tissues were treated with *Agrobacterium* carrying pPOP5 and are under PPT selection.

Medium	No. of explants (weighed in three pooled batches)	Type II callus mg per explant (mean ± SEM)	No. of transgenic shoots
19D - MES	21	14.4 ± 5.8	0
19D + MES	24	32.7 ± 11.0	48
19D + BIS - TRIS	30	19.2 ± 3.4	no data
medium #7	15	29.4 ± 14	no data

#### Example 6.

- 15 We have confirmed the transgenic status of 23 poppy plants in soil, firstly by PAT assay (eg Figure 7). These plants represent at least five independent transformation events. Two of the events are in the cultivar C 058 and three are in Norman. All plants in the glasshouse have flowered and seed has been collected.

Seed ( $T_1$ ) from one line of C 058 and 4 lines of Norman have been sterilised and plated onto medium with and without 10  $\mu\text{g/ml}$  PPT to check the viability of seed, stability of *pat* gene expression and segregation of the transgene. We have shown that the  $T_1$  seed is viable and most progeny have inherited PPT resistance (Table 3).

5 TABLE 3

Line	Total Seed	Abnormal Germination	Healthy Resistant	Susceptible	% R/R+S
Norman	50	4	0	46	0%
Norm 48*1	48	6	19	9	68%
Norm 48*3	48	2	41	0	100%
Norm 48*4	49	1	45	0	100%
Norm 48*5	50	0	14	0	100%

The failure to see susceptible segregants in three lines is not surprising, given that they have multiple inserts.

Seedlings were also germinated and grown without PPT selection and the segregation of PAT enzyme activity has been determined to date on one line (Table 4):

10 TABLE 4

Line	Total Seedlings Tested	PAT +	PAT -	% PAT +
Norm 48*1	27	18	9	67%

We have further confirmed the transgenic status of 9 plants representing at least 5 independent events by Southern blot analysis. Some lines appear to have only a single copy of the *pat* gene, whereas other lines show multiple inserts (Table 5).

TABLE 5

Transgenic Line	Transgenic Event	PAT Transgene Copy Number (Estimate)
Norman 48*1	a	2-4
Norman 48*3 and 4	b	12-20
Norman 48*5 and *6	c	4
C 058 42*1	d	1
C 058 45*9 and *10 and *11	e	1

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contemplated as being within the scope of the present invention.

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